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TITLE: Breast Tissue Dosimetry of PhIP (2-amino-1-methyl-6 phenylimidazo [4, 5b] pryidine) at Human-Relevant Exposures

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The purpose of this research is to define the molecular events leading to the development of PhIP-induced breast tumors and to assess if PhIP exposure at human dietary levels present a human breast cancer risk. Our goals are to understand the effects of chemical exposure on adduct formation and metabolism, the types of adducts formed, how adducts are repaired and the ability of the breast to metabolize PhIP at exposure levels expected to occur via the human diet. To date, we have studied the pharmacokinetics of PhIP in both chronic feeding experiments and accute oral administrations of PhIP. We have continued studies to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups. Additionally, we have investigated the effect of chlorophyllin treatment on the distribution of 14C-PhIP. We are in the process of characterizing the the DNA adducts formed by the PhIP and are determining the effect of dose of PhIp on PhIp-DNA adduct formation in female rodents. We have now synthesized PhIP-DNA for antibody production. Furthermore, tritium AMS methodology has now been developed to the point that a double-labeling experiment can now be performed utilizing 2 different compounds.

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INTRODUCTION
SUBJECT

A great deal of concern has been expressed recently that cooking meat produces genotoxic substances which may contribute to the incidence of human cancers. Of all the substances known to be produced during cooking, the most important may be a class of heterocyclic amines called the imidazoazaarenes (AIA's). These heterocyclic amines are considered to be significant because they are produced at relatively low cooking temperatures such as occur through the grilling, frying, and broiling of red meats, poultry, fish, and grain (1-3). Several of these compounds have also been found in beer and wine and in cigarette smoke condensates (4-6). The AIA's currently identified from cooked foods consist of 19 compounds classified generally as quinolines, quinoxalines, phenylpyridines, and carbolines. All quinoline, quinoxaline and carboline AIAs characterized to date are very potent *Salmonella* mutagens (>100,000 rev/μg). 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a phenylpyridine, is a relatively weak *Salmonella* AIA heterocyclic amine mutagen (2,000 rev/μg), but is the most potent in Chinese hamster ovary cell (CHO) genotoxicity assays (7-9). Other important food-borne carcinogens, such as aflatoxin B_1 or benzo[a]pyrene, are orders of magnitude less potent in genotoxicity assays than the AIAs (10). Importantly, of the 19 known food-borne AIAs, 10 have been tested for carcinogenicity and all ten have been found to induce tumors in both rats and mice; and in multiple organs (2,11,12). Of the AIA's identified, we considered PhIP to be most important since it is present in the highest concentration in well-done beef (2), has been found in cooked grains, beer, wine, and in cigarette smoke; and, unlike most heterocyclic amines, causes breast tumors in the rat (13). Of equal importance, the human exposure of PhIP has been documented as PhIP has been detected in human urine after consumption of normal diets (14,15). Given the recent findings that mutations in the *p53* gene of breast cancer patients are more similar to mutations caused by chemical mutagens than to spontaneous mutations, the role of compounds like PhIP in the etiology of human breast cancer should be critically evaluated (16).

Non-human genotoxicity & metabolism

The mechanism of PhIP's genotoxicity has been most adequately characterized in rodents, but several studies have been carried out in non human primates and human tissue fractions. Understanding these mechanisms is critical to determining if PhIP can cause breast cancer in humans and how to predict an individual's susceptibility. Further, understanding these mechanisms is important since species and tissue specificity in metabolism can ultimately affect the extrapolation of the animal data to humans. PhIP is excreted via the urine and feces, and several stable and unstable DNA- and protein-reactive metabolites have been measured and identified (17-22), although pathways may be dose dependent (23). Pharmacokinetics, metabolism, clastogenicity, and DNA adduct formation have also been measured for PhIP, albeit at exposure levels orders of magnitude greater than found naturally and for tissues other than breast (24-33). Some data have been reported in non-human primates (34-37). The sum of the bioassay data shows conclusively that PhIP is a potent genotoxin and carcinogen. The mutagenicity, and presumably the

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carcinogenicity, of PhIP results from metabolic activation of the parent heterocyclic amine. This principally results from oxidation of the exocyclic amino group to its corresponding N-hydroxylated derivative (2-N-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine) by the cytochromes P450 (23,38,39). The initial oxidation of the PhIP molecule by the cytochromes P450 is followed by one of several conjugations of the exocyclic N-hydroxyl group with acetate, sulfate or other constituents (40-43). Interspecies differences in metabolism have been suggested since rabbit P450IA1 is more active with PhIP than the corresponding P450IA2 whereas human, rat, and mouse P450IA2 is more active than the corresponding P450IA1 (25,43,44). Additionally, N-hydroxy-PhIP is preferentially sulfated in mice (41) and preferentially acetylated in human tissue fractions (Turteltaub *et al.*, unpublished). Such interspecies differences in metabolism may be significant for risk assessment and needs to be understood prior to assessing PhIP's role in human breast cancer. Likewise, the role of the breast in generating bioactive intermediates needs to be understood to develop markers for susceptibility and to understand what makes the breast a target for chemical agents like PhIP.

The principal detoxification pathway for PhIP in rodents and non human primates involves hydroxylation at the 4'-position of the phenyl ring by the cytochromes P450IA (23,35,43,45). The 4'-hydroxyl moiety is subsequently sulfated or glucuronidated to produce several stable excreted metabolites with 4'-PhIP-sulfate [4'-(2-amino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine)-sulfate] being the predominate metabolite detected in plasma, bile and urine (34,35,45,46). Also detected and identified in urine, plasma and bile are the 4'-PhIP-O-glucuronide [2-amino-4'-(b-1-glucosiduronyloxy)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine], and 4'-hydroxy-PhIP (35,45). Glucuronidation of the N^2- and N^3-positions of the imidazole ring system of the N-hydroxylated PhIP molecule [2-(N-b-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine and 3-(N-b-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine, respectively have also been reported (35,42). Analysis of feces has shown primarily 4'-hydroxy-PhIP and PhIP to be present (35,45). These metabolites may be useful in comparing metabolism among species and in predicting susceptibility since they can be easily measured in urine, blood, and breast fluids. The utility of using this approach, however, remains to be determined and will be addressed through this proposal.

The N^2-PhIP-O-glucuronide and the N^3-PhIP-O-glucuronide, like the N:O-acetylated PhIP, may be meta-stable transportable PhIP metabolites. Meta-stable metabolites may serve to cause damage in tissues where PhIP metabolism does not occur. Indeed, such meta-stable metabolites have been suggested as transportable forms of other N-hydroxylamines which are liberated following hydrolysis in extrahepatic tissues (47,48). These metabolites may be causal factors for the DNA damage seen in the blood cells of primates and rodents given PhIP and for DNA and protein damage in tissues where PhIP metabolism does not occur (35). Importantly, PhIP's metabolism has primarily been established using liver tissue fractions and male animals. Few data are available on the metabolism of PhIP in breast tissue or on metabolite levels in breast fluids. These data are needed to understand PhIP's mechanism of action in inducing breast tumors and for understanding if breast fluids can be used in molecular epidemiology studies. The data gathered through this project will specifically fill in these data voids such that the role

of compounds like PhIP in breast cancer can be better understood and used to predict, on an individual basis, who may be at risk. If such an approach proves feasible, it will help be useful in cancer prevention efforts.

DNA and Protein Damage

Exposure to PhIP results in DNA, and likely protein, adduct formation. However, little is known about the identity and sequence specificities of nucleic acid and protein adducts, and in which tissues these most easily form. In addition, tissue specificity in DNA repair is poorly understood. Macromolecular adduction is important since it indicates the active dose of a chemical reaching its target, and is thought to be the initiating event in chemical carcinogenesis. DNA adduct formation with MeIQx has been shown to be quantitatively, but not qualitatively, affected by metabolic capacity (49). PhIP adduct formation may be similarly affected but has not been investigated. N-(deoxyguanosin-8-yl)-3'-monophosphate adducts of IQ, MeIQx and PhIP have been identified and found *in vivo* (36,49-53). Other PhIP adducts also exist and are likewise due to binding at guanines (54). A deoxyguanosin-N^2-yl-PhIP adduct may exist since deoxyguanosin-N^2-yl-MeIQx and MeIQ adducts have been reported.

While most data on the adducts have been derived from studies in the liver, IQ, PhIP and MeIQx have been shown to form adducts in extrahepatic tissues of the rat (12,28,53). High levels of PhIP adducts have been found in the large intestine, white blood cells, pancreas, and heart, followed by stomach, small intestine, kidney, and liver (12,53,55). Some mutational sequence specificity has been demonstrated for *Salmonella* DNA with IQ and PhIP and both inducing GC deletions in the standard frameshift sensitive and uvrB-deficient strains TA98 and TA1538 (56). Protein binding has also been suggested for PhIP (35,57) but, to date, has only been unambiguously demonstrated for IQ (58). A major limitation of the data described above is that all have been derived from high-dose studies and no studies have been reported in the breast even though PhIP causes breast tumors. Thus, little can be determined about the toxicity, biochemistry, and macromolecular targets of PhIP in the breast at human dietary doses.

Human tumorigenesis, genotoxicity, and metabolism

Inadequate data exist on the metabolism and pathologies of all the AIA's, including PhIP, in humans. Several studies have been conducted which show that increased mutagenic activity and some heterocyclic amines can be detected in the urine of fried-meat eaters and men on normal diets, although metabolite recoveries tend to be poor (1, 59-61). Purified human cytochromes P450 and human tissue fractions have been shown to oxidize the AIAs to mutagenic intermediates *in vitro* (62-66). Specifically, liver fractions are known to form the N-hydroxy-PhIP metabolite (67). Further, purified acetyltransferases from human tissues have been used to show that N-hydroxy-PhIP is probably acetylated by the polymorphic arylamine acetyltransferase (68). The paucity of human data can be partially attributed to technical difficulties in measuring metabolism at the low heterocyclic amine concentrations that people are naturally exposed to, and to the difficulty in obtaining material from human subjects. Such difficulty is often

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methodological in nature. A major goal of the work proposed here will be the development and validation of methods which will allow detection of molecular effects in easily accessible human tissues, such as breast fluids and blood. Development and validation of such methods are important for comparing animal and human metabolism, assessing inter-individual differences in metabolism and for eventual use in identifying high risk individuals, since individual differences in metabolism represents a potentially important determinant in risk associated with carcinogen exposure (69).

PURPOSE AND SCOPE OF THE RESEARCH

The scope of this proposal is to determine if the dietary breast carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) causes macromolecular damage in the breast, and the mechanism by which this damage occurs at human levels of exposure. The proposed work will be carried out in female animals for which, as we point out, few data are available. Our purpose is to define the molecular events leading to the development of PhIP-induced breast tumors, and to assess the likelihood that PhIP exposure at human dietary levels present a human breast cancer risk. A crucial step in risk determination is the estimation of the dose of a reactive carcinogen reaching the critical molecular target. DNA adducts are particularly relevant for this purpose since the adduct, if not repaired, can be considered the initial step in the multistage process of cancer. Protein adducts may likewise be useful since they are indicators of the active carcinogen dose in the tissues. Our goals are to understand the effects of chemical dose (exposure) on adduct formation and metabolism, the types of adducts formed, how adducts are repaired, and the ability of the breast to metabolize PhIP at exposure levels expected to occur via the human diet. This low-dose work will be possible by use of AMS, a highly sensitive and novel technique for tracing ^{14}C-labeled xenobiotics with sensitivity in the zeptomole (10^{-21} moles) range. The data collected through this project will help determine if exogenous factors present in the diet can be linked to breast cancer and how best to extrapolate breast cancer risk from standard high-dose tumor assays. Further, this work will lead to a better understanding of the utility of using adducts or metabolites for identifying women at risk for cancer, either because of exposure to high levels of exogenous compounds or due to metabolism genotype. Finally, the data gathered through this work will be used to develop a sensitive assay for assessing PhIP metabolism, exposure and, potentially, risk in humans. If successful, this work will lead in out years to directly studying the molecular epidemiology of PhIP in human breast samples and to defining the role of compounds like PhIP in the etiology of breast cancer.

BODY (PROGRESS TO DATE)

Research completed in the period October 1, 1995 to September 30, 1996 has been very productive, with progress made on all of the specific aims of the project. The progress made towards each of these specific aims are described as follows:

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1. Ultra-low level pharmacokinetics by accelerator mass spectrometry

Towards meeting the goals of specific aim 1 we have administered diets containing ^{14}C-PhIP to rats to achieve daily doses of 10-10,000 ng/kg/day. The goal of these studies is to determine the steady-state levels of PhIP available to the tissues, to determine when adduct levels reach equilibrium in the tissues, and to study the rate of clearance of these compounds. Animals (3 rats/group) have been fed ^{14}C-PhIP containing diets for 6 weeks with twice weekly weighing of the food and animal body weights. In experiments examining clearance rates, the rats were returned to certified rodent chow for an additional 6 weeks. Tissue concentrations of PhIP and PhIP-DNA adduct levels are being measured at selected time points during these studies. AMS analysis of tissues (**Figure 1**) from male rats fed dietary equivalent doses of ^{14}C-PhIP (10 ng PhIP/kg body weight/day), shows rapid accumulation of PhIP in the blood and colon. Steady state levels in the blood were approximately 7 pg PhIP/ml blood while colon levels reached a steady state level of approximately 50 pg PhIP/g colon. After animals were taken off the PhIP-modified diet, tissue clearance rates varied with rapid clearance from the colon and slower clearance from blood. DNA adducts from these tissues are being analyzed and preliminary data show a continual increase in adduct levels through 35 days. A subset of the animals in these experiments were also housed in metabolism cages to assess the absorption and clearance of the administered dose into the urine and feces. For the doses analyzed to date, approximately 5%/day of the dose is eliminated through the urine while approx. 50%/day is eliminated in the feces. This work will continue into year 3 focusing on the mass balance of PhIP in female animals.
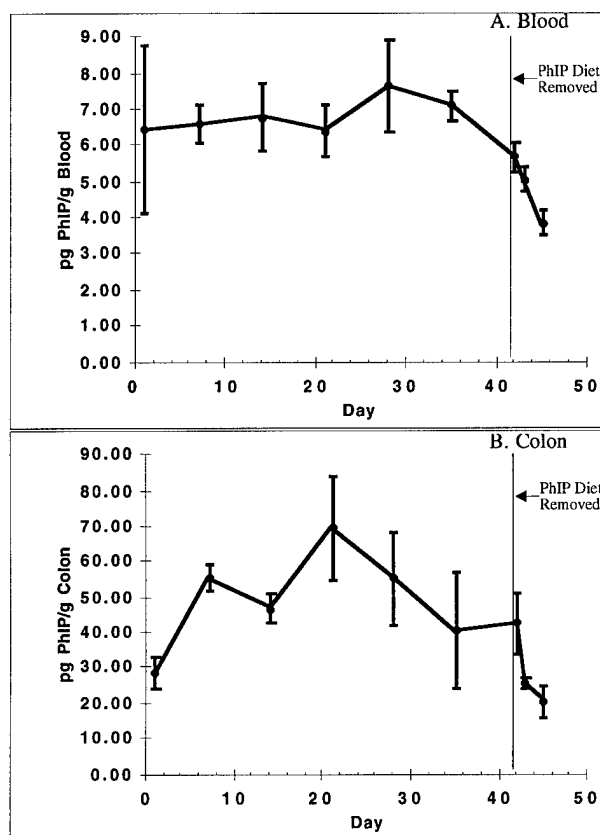
Figure 1. ^{14}C-PhIP concentrations in the blood and colon of rats receiving 10 ng PhIP/kg body weight/day. Data points are the means ± SD of three replicate animals.

In addition to chronic feeding studies, in order to examine the amount of PhIP reaching the breast tissue in female rodents as a function of dose, female F344 rats were acutely dosed by gavage with ^{14}C-PhIP in the dose range 5 ng/kg to 100 mg/kg [36 animals total, 3 animals/dose group], a dose range that incorporates environmentally relevant and rodent bioassay levels. The ^{14}C-PhIP utilized has a specific activity of 10 mCi/mmol, with doses above 10 mg PhIP/kg serially diluted in unlabeled PhIP. Animals were sacrificed 6 hours after dosing, a time point chosen to reflect the initial peak of tissue uptake. Liver, kidney, mammary gland, colon and spleen, which included target and non-target organs for PhIP induced carcinogenicity, were removed and frozen at -20°C until either AMS analysis or DNA extraction. To date, the liver, colon and mammary tissue samples have all been analyzed (**figure 2**).
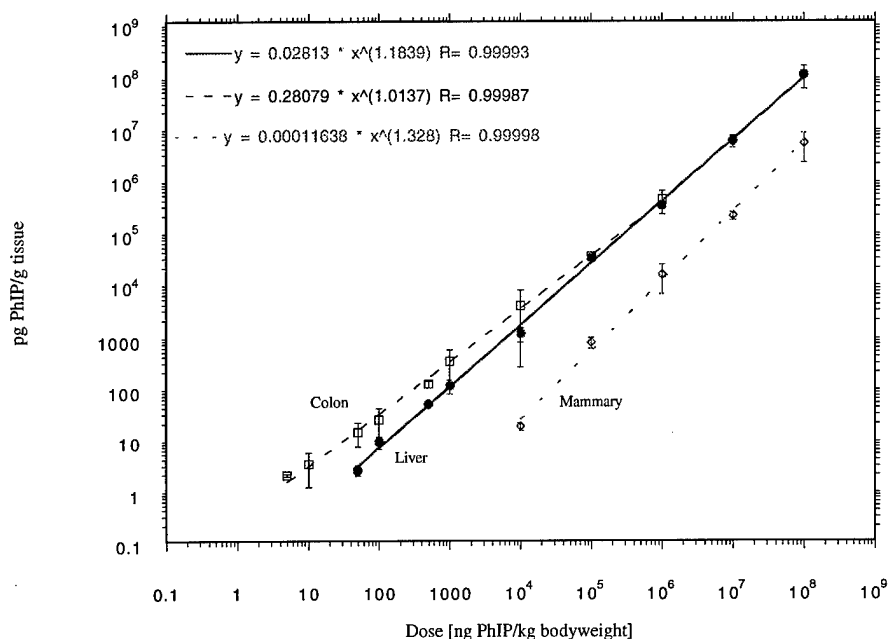
Figure 2. Dose-response for ^{14}C-PhIP in female F344 rats. Data from liver, colon and mammary tissue following a single acute dose are shown. Lines illustrate the power fit and the data points are the means ± SD of three replicate animals.

The concentration of PhIP in the liver, colon and mammary tissue samples increased linearly with administered dose in the dose range 5 ng/kg to 100 mg/kg. The highest level of PhIP in the dose range 5 ng/kg to 1 mg/kg was observed in the colon tissue, followed by the liver. Doses above this were not measured in the colon tissue due to the high levels of ^{14}C, which were too high to measure by AMS. Fundamentally, the mammary tissue contained measurable levels of PhIP. The mean mammary tissue levels were 4.63 x 10^6 pg PhIP/g tissue at 100 mg/kg dose and 17 pg PhIP/g tissue at 10 μg/kg dose. Below 10 μg/kg binding was not detectable in the mammary tissue, which may partially be due to the very high levels of carbon in this tissue [67.7% carbon] compared to the liver [29.7% carbon] and colon [11.8% carbon], which would effectively dilute any ^{14}C signal. Initially, combustion of mammary tissue samples prior to AMS measurement was problematic, as combustion tubes frequently exploded. This was considered to be due to the very high carbon levels which resulted in an excessive amount of CO_2 being produced during the combustion process. Further analyses were completed with reduced amounts of mammary tissue compared to liver and colon samples.

In conclusion, the acute PhIP dosing study has demonstrated that PhIP is distributed to both the colon and mammary tissue, the target organs for PhIP-induced carcinogenicity in female rats. Furthermore, the levels of PhIP in the mammary and colon tissues increase as a linear function of dose. In the coming year we will compare the dose response in the females to males in

order to understand bioavailability among the sexes and to see if it relates to to PhIP-induced tumor sensitivity.

2. Breast metabolism of PhIP

Towards specific aim 2 we have conducted a study to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups. Additionally, we are developing HPLC/AMS separation protocols for determination of metabolite levels in the milk from these animals. Lactating female F344 rats with suckling pups were gavaged with doses ranging from 50-1000 ng/kg ^{14}C-PhIP. The excretion of the ^{14}C-PhIP in the milk and distribution of ^{14}C-PhIP into the mammary tissue, liver and blood of the dam as well as in the stomach contents, liver and urine of their suckling pups were measured using AMS (**Figure 3**).
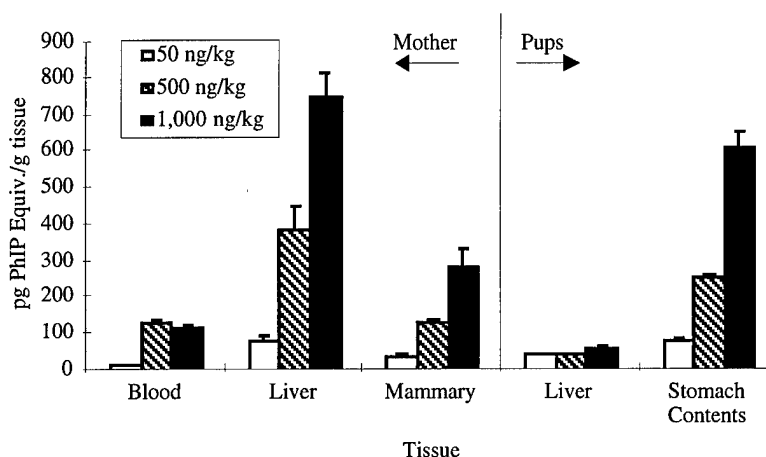
Figure 3. Distribution of ^{14}C-PhIP in lactating female rats and their suckling pups. Data points for Dams are the means ± SD of three replicate animals. Data points for the pups are from three individual pools of pup litters.

^{14}C-PhIP derived radioactivity increased in a dose-dependent manner in both the milk and stomach contents of the pups, as well as in the other tissues measured. Highest levels of PhIP related radioactivity were found in the liver with levels of approximately 750 pg PhIP equivalents/g liver tissue for the 1000 ng/kg dose. Although significantly lower, the mammary tissue contained high levels of PhIP derived material with levels of approx. 300 pg/g mammary tissue at the 1000 ng/kg dose. In an effort to examine potential chemopreventive therapies, lactating female rats also were dosed with 500 µg/kg chlorophyllin in conjunction with a 500 ng/kg ^{14}C-PhIP dose. The chlorophyllin treatment caused increased levels of ^{14}C-PhIP in the milk and stomach contents of the pup while decreasing levels in all other tissues measured (**Figure 4**) HPLC/AMS analysis of both the metabolites found in the milk and pup urine will be analyzed for differences caused by the additional chlorophyllin treatment. The results from these studies suggest that at dietary levels of PhIP, PhIP and PhIP metabolites are excreted into the breast milk and absorbed by the newborn. The findings raise the possibility that there is a carcinogenic risk to the newborn by exposure to normal dietary levels of PhIP

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via the breast milk. The addition of chlorophyllin to the dosing regimen demonstrates that other components of the diet modulate the excretion of ^{14}C-PhIP-derived radioactivity into the breast milk and alter the uptake into tissues of newborns. The effects of addition of chlorophyllin has implications for chemoprevention strategies. Additional work on analysis of PhIP metabolites in the milk and pup urines is underway and a manuscript on these results in in preparation and should be submitted in the next few months.
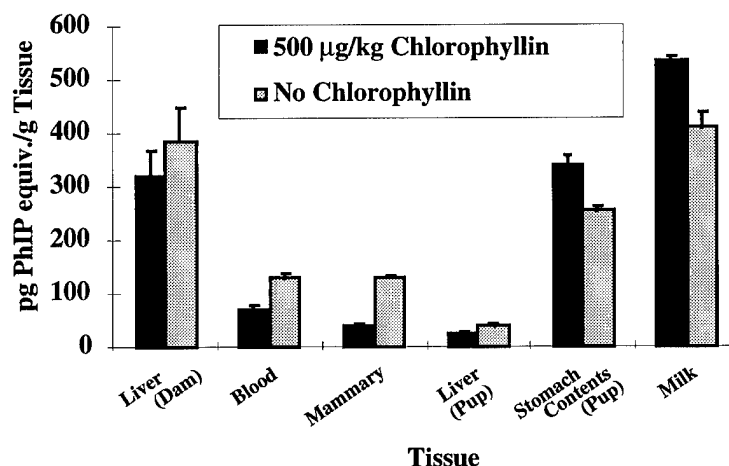

Figure 4. Effect of 500 mg/kg chlorophyllin cotreatment with 500 ng/kg ^{14}C-PhIP treatment. Data points are the means ± SD of three replicate animals.

3. Determination of DNA and protein adducts of PhIP in the breast

The third objective of these studies is to determine if protein and DNA adducts are formed in the breast tissue of female rodents dosed with PhIP. In addition, characterization of the PhIP-DNA adducts formed is to be undertaken using mass spectrometry and NMR technology in comparison to synthetic PhIP-DNA adducts. To date, only 1 DNA-adduct has been structurally identified, although several more may form *in vivo*. Therefore, there is a requirement for chemically synthesized adducts which will be employed as standards.

During the last year, much progress has been made in the production and characterization of synthetic PhIP-DNA adducts to be used in the identification of the DNA adducts formed *in vivo* in rats dosed with PhIP. Synthetic DNA adducts have been produced by the reaction of N-hydroxy PhIP and N-acetoxy PhIP with calf thymus DNA and deoxyguanosine and the spectra of adducts produced analyzed by absorption and fluorescence spectroscopy, as well as ^{32}P-postlabeling (73). It was established that DNA modification by N-hydroxy PhIP was 20-50 times less efficient than by the further esterified N-acetoxy PhIP, although the spectra of adducts produced by both appeared identical. Primarily dG-C8-PhIP adduct was formed by both species, with small yields of 3 other adduct species. Adduction in MOPS or phosphate buffers (pH 6.5-7.0) compared to citrate buffer (pH 5.0) resulted in greater yields of the 3 minor adducts compared to the dG-C8-PhIP adduct. Furthermore, these adducts were consistent with the DNA adducts observed in PhIP dosed animals. A fourth adduct was detected in N-hydroxy PhIP modified DNA, which was not seen in N-

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acetoxy PhIP modified DNA or *in vivo*. Therefore, as the adducts formed *in vivo* appear to be the same as those formed *in vitro*, we will be able to use these standards to identify which of the adducts are formed by PhIP in breast tissue. This work has been submitted for publication and is under review at the present time.

Further work is underway to fully characterize the minor DNA adducts present in N-acetoxy PhIP modified DNA. In order to do this, bases, deoxyribonucleosides and deoxyribonucleotides are being adducted and analyzed by mass spectrometry. Data to date, suggests PhIP forms one major adduct which we have confirmed as the dG-C8 guanine adduct, and 4 minor adducts seperable by HPLC. Each of the minor adducts has an m/z of 507 but unique fragmentation pattens by ES-MS analysis. Initial data sugests one is a ring opened adduct. As part of this investigation we have obtained heavy isotope labeled guanine in the form of $[4,5,6,8-^{13}C_4][9-^{15}N]$ guanine from Dr. Ian Blair (Vanderbilt University), which will aid interpretation of the adduct fragmentation patterns. Future experiments will use the labeled guanine to produce deoxyguanosine in adduction experiments, which will more accurately reflect the reactions that occur with DNA.

In preparation for NMR studies, adduction experiments are now being conducted utilizing an 11 base pair double-stranded oligonucleotide containing a 5'-GGA-3' 'hot-spot' for PhIP modification. The conformations of adducts in N-acetoxy PhIP adducted oligonucleotide will be determined by NMR spectroscopy using a 600 mHz Varian spectophotometer. To date, an oligomer has been synthesized and its sequence confirmed by NMR. Reactions to modify the oligomer will begin shortly. Based on preliminary studies we expect modification levels on the order of 15 - 20%, which has been sufficient for solution structure determinations of other adducts. The solution structure determination will be carried out by Dr. Monique Cosman (LLNL).

The improved HPLC-based postlabeling assay we have developed has also been used for measuring the individual PhIP-DNA adducts in animal models. With HPLC Inline Precolumn Postlabeling (HIPP) and a sensitivity of approximately 1 adduct/10^9 bases, we are able to measure PhIP-DNA adducts in animal models, cell culture systems, and tissue slices. Postlabeled samples are loaded onto a C_{18} precolumn and adducted bases are retained while excess radioactivity and the unmodified bases are eluted through a UV detector to waste through a switching valve. The use of this inline precolumn enrichment allows entire postlabeled samples to be analyzed without prior purification of labeled adduct and also allows determination of the exact amount of sample loaded onto the column. The adducted samples are then eluted onto an analytical reversed phase column to separate the individual PhIP-DNA adducts **(Figure 5)**.
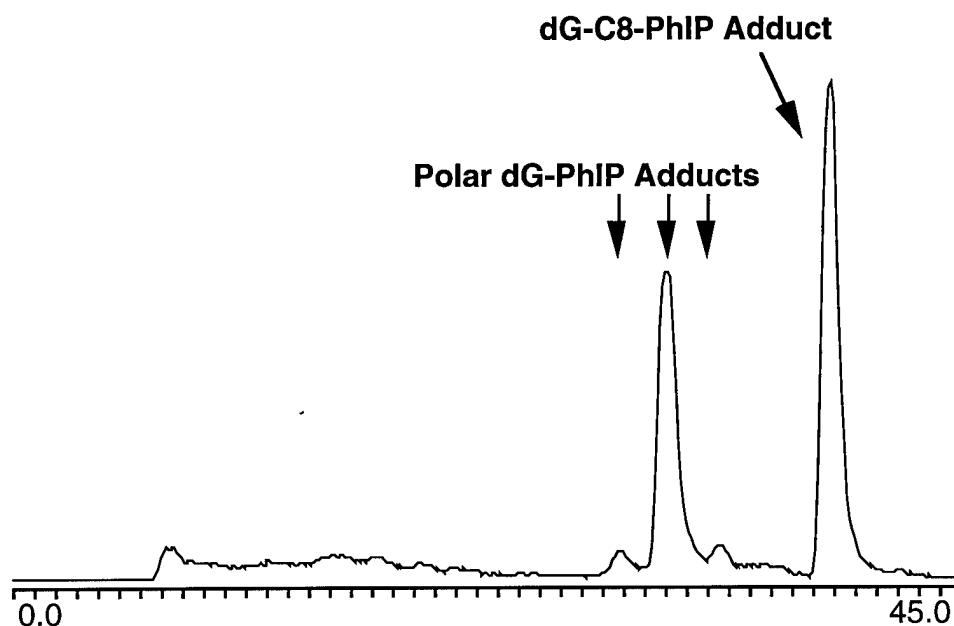
dG-C8-PhIP Adduct

Polar dG-PhIP Adducts

0.0　　　　　　　　　　　　　　　　　　　　　45.0

Figure 5. Typical ^{32}P-postlabeling profile for PhIP modified DNA using newly developed HPLC Inline Precolumn Postlabeling (HIPP) methods.

PhIP-DNA samples show 2 major peaks and up to three additional minor adduct peaks when labeled under ATP-limiting conditions. The method has a sample to sample standard error of 10 percent at adduct levels of 1 adduct/10^7 bases and shows a linear relationship between signal and adduction levels down to approximately 1 adduct per 10^9 bases. Individual DNA samples (1 to 25 μg) can be analyzed by HPLC in less than 1 hour allowing high throughput of postlabeled samples. Extensions of this technique designed to measure overall adduct levels rather than separate specific PhIP-DNA adducts allows for 15 minute analysis times (rapid-HIPP). These short analysis times allow for more replicates to be measured yielding higher accuracy and precision in the measurements. For example, DNA adduct levels in a study where rat colon slices were exposed to N-OH-PhIP were determined with less than 10% errors using the rapid-HIPP method **(Figure 6)**. These extremely accurate postlabeling values are typically very difficult to achieve using standard postlabeling assays and allows for a much wider variety of studies to be performed. In addition to the high resolution provided by HPLC separation of the PhIP-DNA adducts, this method can be adjusted for analysis of other DNA adducts and is readily automated for high throughput and decreased handling of ^{32}P. We anticipate application of this assay to measurement of adducts in humans in later years. A manuscript describing this technique has been published (74).
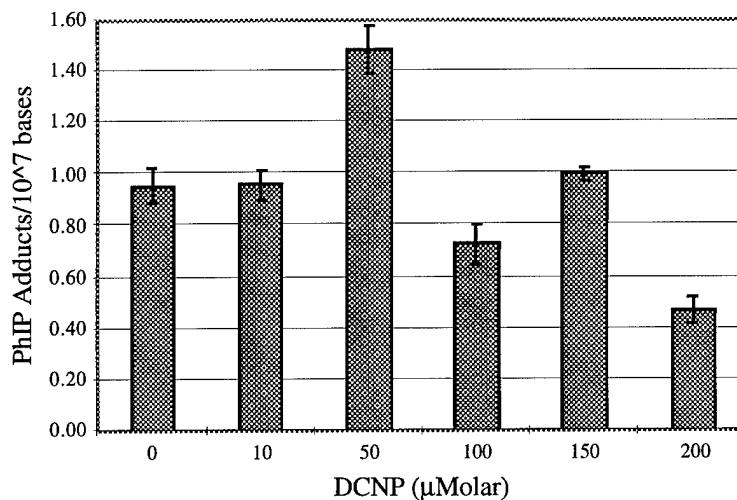
12

PhIP Adducts/10^7 bases

1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00

0   10   50   100   150   200

DCNP (μMolar)

Figure 6. ^{32}P-postlabeling results demonstrating high accuracy of rapid-HIPP assay. Errors average approx. 6%. In this case, colon slices were incubated with N-OH PhIP and increasing concentrations of DCNP, a phase II conjugation inhibitor (Malfatti et al., (1996) Cancer Research vol 56, 2550-5).

4. Dose-response relationships

Objective 4 is concerned with the understanding of the effects of the dose of PhIP on DNA adduct formation in breast and non-breast tissues, hence establishing data to be employed for the extrapolation to breast cancer risk in humans at low-dose exposures.

As part of these studies, the dosimetry of PhIP on PhIP binding to the DNA in the liver, colon and mammary tissue is being determined in an acute PhIP dosing study [for details of the dosing regimen refer to objective 1]. DNA from liver, colon and mammary tissue samples in this study were extracted and the covalent ^{14}C-PhIP binding measured by AMS. Initial difficulties have necessitated the investigation of additional purification steps in the DNA extraction procedure. Furthermore, liver DNA initially provided low ^{14}C signal requiring adapation of the sample preparation methods so that undiluted DNA could be measured [which requires approximately 500 μg DNA]. These new methods increase the sensitivity and accuracy of the measurement and allow a maximum sensitivity of approximately 1 adduct/10^{11} nucleotides (with 10 mCi/mmol ^{14}C-PhIP). Unfortunately, this methodology could not be utilized with mammary DNA due to the relatively low yields of DNA [0.1-0.3 mg DNA/g tissue]. Analysis of the mammary DNA with a reduced amount of tributyrin carrier, but sufficient for graphitization, is currently in progress. The results of the colon and liver DNA analyses are shown in **figure 7**.
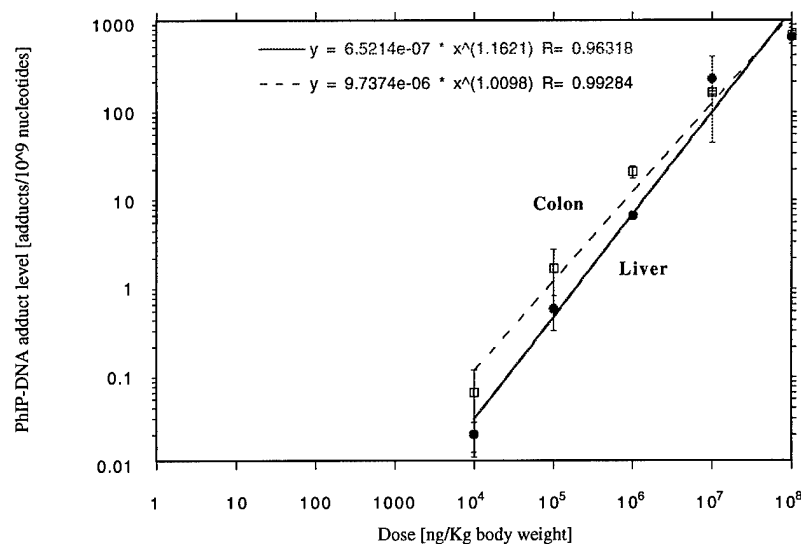
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Figure 7. Dose-response curves for DNA adduct formation by ^{14}C-PhIP in female F344 rat liver and colon following a single acute dose. The power fit lines are shown. Data are means $\pm$ SD of three replicate animals.

Analogous to the tissue binding data, the dose-response curves for DNA adduct formation in the liver and colon were linear over the measurable range with a mean peak adduct level at 10 µg/kg dose of 6.0 and 2.0 adducts/10^{11} nucleotides in the colon and liver respectively. DNA adducts were not detectable in either the liver or colon at doses below 10 µg/kg using the acute dosing regimen.

5. Development of an AMS-Isotope-labeled Immunoassay

The purpose of specific aim 5 is to produce antibodies against PhIP modified DNA. The antibodies will then be utilized in a selective and sensitive immunoassay to detect and quantify PhIP-DNA adducts in various biological samples from laboratory animal and human studies. The immunoassay will potentially have applications in a wide range of molecular epidemiology studies to investigate the link between breast cancer and PhIP exposure. For example, in the validation of PhIP-DNA adducts as biomarkers in assessing exposure to PhIP and in determining susceptibility to breast cancer. In addition, it may also be useful for assessing the effectiveness of cancer chemopreventive agents.

In order to reach the goals of specific aim 5, methods established by Marsch *et al.* (73) for the modification of DNA by PhIP have been utilized in order to obtain a DNA modification level sufficient for antibody production [approximately 1 adduct/100 nucleotides] in sufficient quantity [10-15 mg DNA]. In order to achieve this high level of modification with a large amount of DNA, the scale of the reactions had to be greatly increased.

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N-acetoxy PhIP [considered to be the ultimate DNA binding species] was synthesized by firstly converting PhIP to N-hydroxy PhIP. The resultant N-hydroxy PhIP was N:O acetylated by addition of acetic anhydride. The product, N-acetoxy PhIP [in a ratio of 1:10, N-acetoxy PhIP: nucleotides], was then added dropwise to a 4 mg/ml solution of thoroughly degassed calf thymus DNA in 50mM sodium citrate pH 5. The reaction was continued for 2 hours at room temperature with stirring under an atmosphere of nitrogen gas. The modified DNA was then purified by repeated extraction with water saturated 1-butanol, followed by chloroform and then finally precipitation with ethanol. The DNA appeared bright orange in color [characteristic of highly modified PhIP-DNA]. The DNA was redissolved in phosphate buffer pH 7 and then dialyzed for 3 days at 4°C with 3 changes of buffer. Finally, the UV absorbance spectrum was measured **(figure 8)** and DNA concentration determined in order to calculate the modification level. The modification level was calculated to be 1 adduct/115 nucleotides, which was judged sufficient for antibody production. 15 mg of the DNA was stored frozen for shipment to Dr. Miriam Poirier [NIH] for antibody production and the remaining DNA was kept for analysis of the adducts.
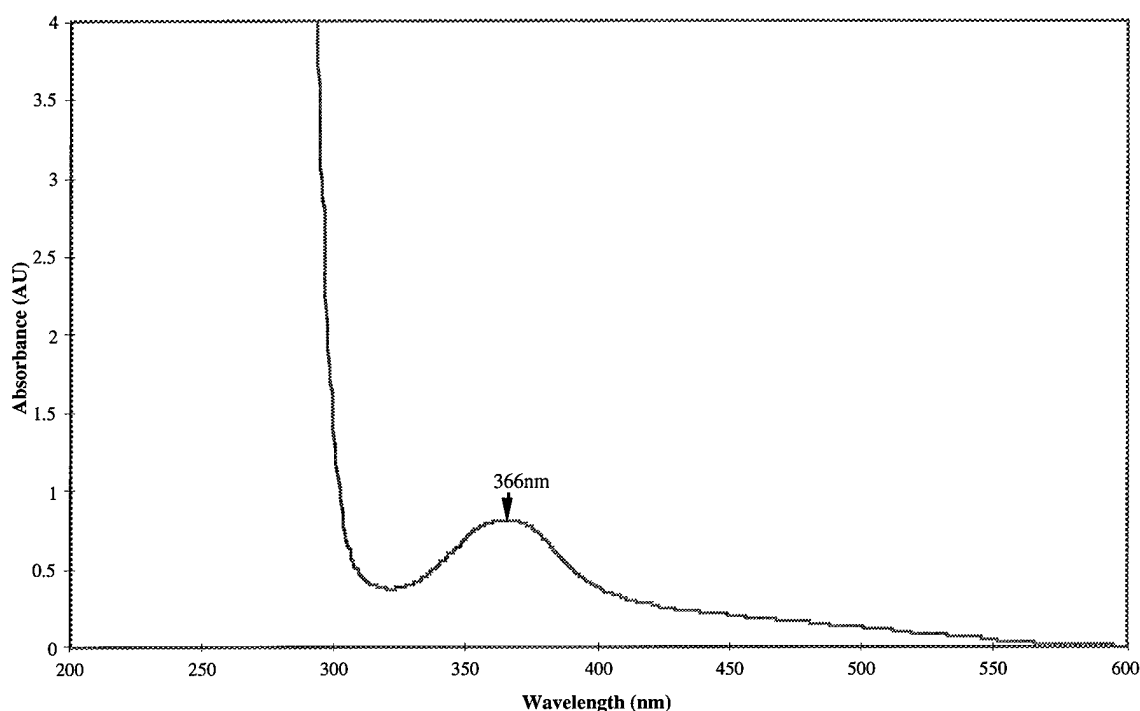

Figure 8. UV absorbance spectrum of N-acetoxy PhIP-modified DNA at pH 7. The PhIP-DNA adduct absorbance maximum is 366 nm.

In order to investigate if the modification of the DNA could be further increased by repeated modification with N-acetoxy PhIP, the above reaction was performed twice on a single sample of calf thymus DNA. The resulting modification level was 1 adduct/77 nucleotides [6 mg total DNA], however, the UV absorbance spectrum revealed 2 peaks of adduct absorbance, which was not observed with the DNA modified only once. This sample was also analyzed by [32] P-postlabeling to investigate the adduct distribution.

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Analyses of the adduct distribution of both adducted samples were performed by ^{32}P-postlabeling using both TLC and HPLC separation. The HPLC-based postlabeling method employed has been developed in this laboratory (74) and enables sensitive measurements to be made in less than an hour. The spectrum of adducts observed when both adducted samples were analyzed were similar to those previously reported by Marsch *et al.* (73) for PhIP modified DNA. Three main adduct peaks were detected, the largest one having a retention time that corresponded to the dG-C8 PhIP adduct. Therefore, the DNA from both adduction reactions was sent to Dr. Poirier for production of polyclonal antibodies. The immunization of rabbits is scheduled to commence in October 1996.

Methods of Procedure used in these studies: Development of tritium AMS

Tritium-Accelerator Mass Spectrometry (^{3}H AMS) is being developed in order to measure the ^{3}H content of mg-sized biological research samples. LLNL has already successfully applied ^{14}C AMS to a variety of problems in the area of biomedical research and the development of ^{3}H AMS would complement these studies. The ability to perform ^{3}H AMS measurements at sensitivities equivalent to those obtained for ^{14}C will make it possible to perform experiments using compounds that are not readily available in ^{14}C-tagged form. In addition, unique double-labeling experiments could be performed in which the fate, distribution, and metabolism of separate fractions of biological compounds could be studied. For example, tritiated compounds could be utilized to establish DNA repair rates in conjunction with ^{14}C-PhIP exposures.

^{3}H AMS methodology has already been partially developed at the Lawrence Livermore Laboratory (76). However, in order to investigate the accuracy of ^{3}H AMS compared to ^{14}C AMS for the detection and quantification of tracers in biological systems, mice were dosed with equimolar quantities of 0.01-10 mg/kg ^{3}H and ^{14}C labeled PhIP by gavage. After 1 hour, the mice were sacrificed and the livers frozen until analysis. Liver tissue samples were analyzed in replicates of 4 by ^{14}C AMS for ^{14}C-PhIP content, in parallel to preparation for ^{3}H AMS analysis. For ^{3}H AMS, the optimal tissue size was 5 mg, which was then thoroughly dried by centrifugal evaporation in order to remove water. In addition, tributyrin standards are used with known amounts of ^{3}H which have been analyzed by scintillation counting. The correlation between data obtained by ^{3}H and ^{14}C AMS are shown in **figure 9**.
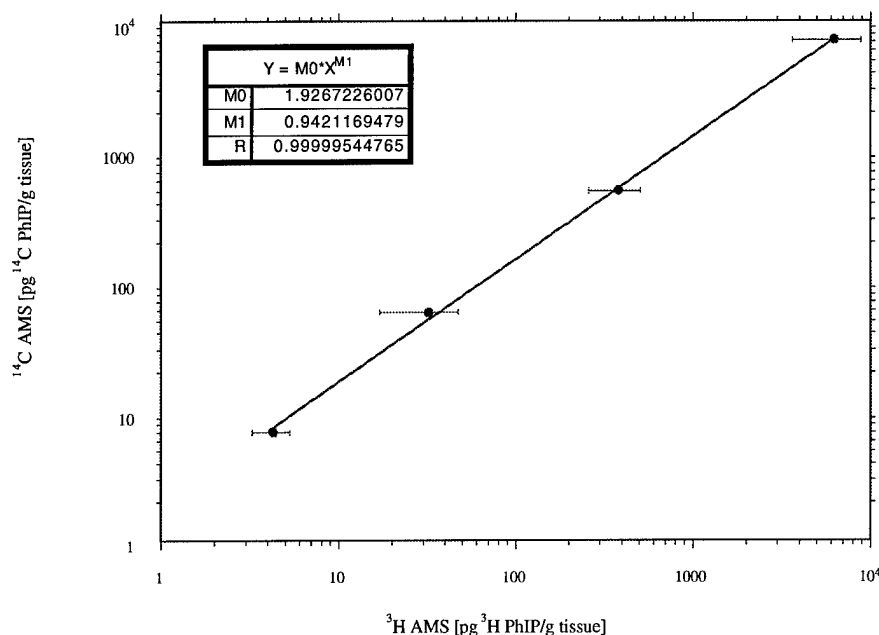
Figure 9. Plot to illustrate the correlation between PhIP levels in mouse liver quantified by ^{3}H and ^{14}C AMS. The power fit of the data is shown. Data points are the means ± SD of 4 replicates [^{3}H AMS] and 3 replicates [^{14}C AMS]. Note: Error bars for ^{14}C AMS are plotted.

The data indicated a good correlation between ^{3}H and ^{14}C AMS for the measurement of PhIP in liver tissue [by regression analysis $p<0.05$, $r^2=0.999$], although the ^{3}H AMS revealed standard deviations greater than observed with ^{14}C AMS [an average of 34% of the mean compared to less than 10% with ^{14}C AMS]. More recent experiments have concentrated upon reducing these uncertainties, by optimiziation of sample preparation parameters. An experiment is now planned to use the methodology in the first double-labeling experiment utilizing 2 different compounds. We antipate preparing methods paper for the analysis of tritium by AMS later this year.

CONCLUSIONS

To date, a significant amount of progress has been made in our specific aims.

We have studied the pharmacokinetics of PhIP in both chronic feeding experiments and acute oral administrations of PhIP in accordance with specific aim 1. We have included both male and female F344 rats to provide useful comparisons between gender and to credibly assess our results versus published data performed with higher doses. In these studies we have been able to demonstrate dose-response trends for tissue concentrations of PhIP and

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DNA adduct levels. Initial difficulties in working with mammary tissue have been resolved.

We have continued studies to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups in accordance with specific aim 2. Additionally, we have investigated the effect of chlorophyllin treatment on the distribution of ^{14}C-PhIP. These studies have revealed that even at low human dietary equivalent doses, PhIP and PhIP metabolites are passed to sucklings pups and may pose a carcinogenic risk to the pups. Further, while chlorophyllin appears to be a reasonable detoxifying agent for the dams, it actually increases the exposure of the pups to PhIP. The results from these studies are being prepared for publication while characterization of the metabolic profiles of PhIP metabolites in the milk and urine of the pups continues. Work using mammary tissue homogenates is scheduled to begin within year 3 of this grant.

We are in the process of characterizing the DNA adducts formed by PhIP in accordance with specific aim 3. The chemistry to optimize PhIP-DNA adduct formation has been performed and adduct sysnthesis can be directed towards either mainly C-8 adduct or towards the uncharacterized polar adducts. These adducts have been compared to *in vivo* adducts using ^{32}P-postlabeling. In addition, a portion of these polar adducts have ben characterized by triple-quadrapole mass spectrometry, UV absorbance and fluorescence spectroscopy.

We are determining the effect of dose of PhIP on PhIP-DNA adduct formation in female rodents in accordance with specific aim 4. Acute oral exposures to female F344 rats have been performed and linear dose-response relationships observed. Therefore, even at low dietary relevant doses, DNA adducts are formed and therefore may be involved in the carcinogeneic effects of PhIP. This data supports the role of PhIP in breast cancer as indicated from epidemiological studies linking the consumption of food likely high in heterocyclic amine content with breast cancer.

We have now synthesized PhIP-DNA for antibody production in accordance with specific aim 5. This DNA will be used to produce antibodies early in year 3 of this grant. Over the remainder of year 3, the antibodies will be tested and characterized. Furthermore, tritium AMS methodology has now been developed to the point that a double-labeling experiment can now be performed utilizing 2 different compounds. This will allow a variety of experiments to be performed, including DNA repair assays and synergism studies.

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